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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 48/00, C07H 21/04, C12N 15/63, 15/85</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/34638</b> <b>(43) International Publication Date:</b> 25 September 1997 (25.09.97)
<b>(21) International Application Number:</b> PCT/US97/04519 <b>(22) International Filing Date:</b> 20 March 1997 (20.03.97)  <b>(30) Priority Data:</b> 60/013,796 20 March 1996 (20.03.96) <b>US</b>  <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). <b>(72) Inventor:</b> FELDMAN, Lawrence, T.; 224 9th Place, Manhattan Beach, CA 90266 (US). <b>(74) Agent:</b> BERLINER, Robert; Robbins, Berliner & Carson, L.L.P., 5th floor, 201 N. Figueroa Street, Los Angeles, CA 90012-2628 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ANTISENSE APPROACH TO GENE INHIBITION  <b>(57) Abstract</b>  The present invention discloses splicing cassettes, vectors, and transformed cells that enable the production of stable introns having antisense sequences. The stable introns are produced during the RNA splicing procedure, wherein RNA lariats are formed having a branch point that cannot be debranched. When introduced into the nucleus of eukaryotic cells, the stable antisense RNAs will inhibit the expression of complementary mRNAs. This inhibitory effect forms the basis of a method of gene therapy that can be used to treat a number of infectious diseases and cancers.		

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## ANTISENSE APPROACH TO GENE INHIBITION

### BACKGROUND OF THE INVENTION

The concept of antisense-mediated gene inhibition was introduced by Stephenson and Zamecnik using oligodeoxynucleotides {Stephenson, M. L. *et al.*, *PNAS (USA)*, **75**:285-288 (1978)}. These

- 5 oligodeoxynucleotides tend to be chemically synthesized, and short, on the order of 5-30 bases. There are problems associated with the uptake of these molecules by the cell, and with their specificity, since they are so short.

- 10 An approach which overcomes some of these problems is the use of a promoter to synthesize an antisense RNA molecule. These RNAs are typically capped and polyadenylated just like mRNAs, and are transported to the cytoplasm. While these RNAs are longer, they are often only partially effective because they are not abundant enough to inhibit all the mRNA, or, because of their cytoplasmic location, they do
- 15 not inhibit the mRNA soon enough, and too high a percentage of message is translated into protein. A variation of this antisense approach is to use a ribozyme as part of the RNA structure so that the ribozyme is targeted specifically to the mRNA. This is an interesting approach, but the ribozyme targets are very short sequences, making
- 20 inhibition of a wide array of mRNA difficult.

Jacquier, *et al.* {Jacquier, A., *et al.*, *PNAS(USA)*, **83**:5835-5839 (1986)} summarized recent studies in a number of laboratories which have clarified the pathway by which pre-mRNA processing (splicing) takes place. In both yeast (*saccharomyces cerevisiae*) {Pikielny, C. W.,

*et al.*, *Cell*, **34**:395-403 (1983); Domdey, H., *et al.*, *Cell*, **39**:611-621 (1984); and Rodriguez, J. R., *et al.*, *Cell*, **39**:603-610 (1984)) and mammalian cells {Grabowski, P. J., *et al.*, *Cell*, **37**:415-427 (1984); Padgett, R. A., *et al.*, *Science*, **225**:898-903 (1984); Ruskin, B., *et al.*, *Cell*, **38**:317-331 (1984); and Zeitlin, S., *et al.*, *Cell*, **39**:589-602 (1984)), a two-step process has been described. First, 5' splice-site cleavage and lariat (branch point) formation takes place, in which the 5' end of the intron is attached to the branch point via a 2'-5' phosphodiester bond. In yeast, the last adenosine of the "TACTAAC box" serves as the branch point, while in Metazoa the branch point sequence is more variable and is specified in part by distance from the 3' splice site. Second, 3' splice-site cleavage and exon ligation take place, during which the lariat intron is released. Both step 1 and step 2 may be coupled cleavage-ligation events, as neither 5' cleavage nor 3' cleavage has been detected in the absence of lariat formation or exon ligation, respectively. It appears that these processes take place predominantly or entirely in large ribonucleoprotein particles in which pre-mRNA substrate is localized after addition to a splicing extract {Brody, E., *et al.*, *Science*, **228**:963-967 (1985); Frendewey, B., *et al.*, *Cell*, **42**:355-367 (1985); and Grabowski, P. J., *et al.*, *Cell*, **42**:345-353 (1985)}.

Alternative splicing of a particular primary transcript can give rise to a variety of mRNA species, each encoding a different protein. Also, it has been suggested that the splicing of introns from primary transcripts may facilitate transport of the mRNA from the nucleus to the cytoplasm {Buchman, A. R., *et al.*, *Mol. Cell. Biol.*, **8**:4396-4405 (1988)}.

The latency-associated transcript (LAT) is the major viral transcript detected by in situ hybridization of mouse and human sensory ganglia latently infected with herpes simplex virus type 1. The last 750 bases of LAT are complementary, that is, antisense, to infected-cell polypeptide 0, a herpes simplex virus type 1 (HSV-1) immediate-early gene that encodes a transactivating protein that may facilitate re-activation of the

virus from the latent state. LAT effectively inhibits transactivation of gene expression by infected-cell polypeptide 0 in transient transfection assays.

### **SUMMARY OF THE INVENTION**

5       The present invention is directed to cassettes, vectors, and transformed cells that enable the production of stable introns having antisense sequences. The stable introns are produced during the RNA splicing procedure, wherein RNA lariats are formed having a branch point that cannot be debranched. When introduced into the nucleus of  
10 eukaryotic cells, the stable antisense RNAs will inhibit the expression of complementary mRNAs. This inhibitory effect forms the basis of a method of gene therapy, that can be used to treat a number of infectious diseases and cancers.

      The invention specifically provides for splicing cassettes which  
15 facilitate the molecular cloning of stable antisense introns. The splicing cassettes contain: a) a promoter, capable of expressing RNA sequences in eukaryotic cells; b) a splice donor site downstream from the promoter; c) a splice acceptor site downstream from the splice donor site; d) a branch point sequence between the splice donor and acceptor sites,  
20 wherein the branch point can form a lariat but cannot debranch; and e) an antisense sequence inserted between the splice donor site and the branch point sequence.

      Preferred embodiments of the splicing cassette have promoters selected from the group consisting of the CMV immediate early promoter,  
25 a T cell specific promoter, a retrovirus LTR, and a promoter that functions solely in CD4 positive T cells.

      Other preferred embodiments of the splicing cassette have branch point sequences derived from the herpesvirus LAT sequence or T cell receptor sequences.

30       Particularly preferred embodiments of the splicing cassette have antisense sequences complementary to mRNA sequences of HIV,

Hepatitis B & C, Epstein Barr virus, Varicella Zoster Virus, Cytomegalovirus, Herpes Simplex Virus, and TGF- $\beta$ .

Another embodiment of the present invention is an antisense vector, which is a transfer vector capable of transducing or transforming eukaryotic cells and a splicing cassette inserted within the transfer vector. Preferred versions of the antisense vector use transfer vectors, such as retroviral, adenoviral, and adeno-associated viral vectors.

The present invention further provides for cell lines, which are eukaryotic cells stably transformed by an antisense vector. The antisense vector can then express a stable intron within the nucleus of the transformed eukaryotic cells. Preferred versions of the cell line include eukaryotic cells, such as SUPT1, CD34 cells, CD4 cells, hematopoietic stem cells, and hematopoietic progenitor cells.

The invention also provides for a method of gene therapy, wherein eukaryotic cells are transformed or transduced with an antisense vector. The eukaryotic cells can be CD34 cells, CD4 cells, hematopoietic stem cells, hematopoietic progenitor cells, bone marrow cells, liver cells, or neurons. Moreover, the genetic modification of eukaryotic cells by the antisense vector may occur either *ex vivo* or *in vivo*.

The present invention is directed to cassettes, vectors, and transformed cells that enable the production of stable introns having antisense sequences. The stable introns are produced during the RNA splicing procedure, wherein RNA lariats are formed having a branch point that cannot be debranched. When introduced into the nucleus of eukaryotic cells, the stable antisense RNAs will inhibit the expression of complementary mRNAs. This inhibitory effect forms the basis of a method of gene therapy, that can be used to treat a number of infectious diseases and cancers.

Figure 1 diagrams the processes of (A) normal lariat formation and degradation during splicing and (B) lariat formation without debranching when there is a faulty branch point;

Figure 2 is a pictorial representation of the processes of (A) normal lariat formation, debranching, and degradation during splicing and (B) lariat formation without debranching when there is a faulty branch point;

Figure 3A is a diagram of the structures of spliced HIV mRNAs;

Figure 3B shows where the antisense constructs of the examples overlap with HIV mRNAs; and

Figure 4 presents the results of an RNase protection assay, which shows presence of stable antisense intron RNA in cells transfected with antisense vectors.

#### DETAILED DISCUSSION OF THE INVENTION

The abbreviations used in this application are as follows:

AIDS	acquired immunodeficiency syndrome
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
LAT	latency associated transcript
mRNA	messenger RNA
RNA	ribonucleic acid
SCID	severe combined immunodeficiency

In considering an antisense approach to HIV infection, for example, the prior art oligodeoxynucleotides would be too short, too toxic, and not specific enough to inhibit many viral strains of HIV. In addition, antisense oligonucleotides do not enter the nucleus and do not replicate with dividing cells, thus limiting their use. Similarly, many HIV isolates would not be attacked by a specific ribozyme, and others could

easily mutate the ribozyme site to avoid digestion. An antisense mRNA approach might work, but the cytoplasmic location of the antisense mRNAs would be a disadvantage once the virus begins its lytic cycle.

In eukaryotic cells, the intron between two exons is spliced by  
5 endonucleolytic cleavage at the splice donor and splice acceptor sites. The 5' end of the intron then binds to the branch point, which has the consensus UACUAAC, with binding to the third A. By binding to the branch point, which is 20 to 30 bases from the 3' end of the intron, a lariat is formed.

10 As shown in Figs. 1A and 2A, under normal conditions, this lariat is de-branched by a cellular enzyme. This leaves the 5' end of the intron exposed to potent ribonucleases which degrade the intron. In Herpes Simplex Virus, there is the LAT, which is actually a stable intron. It appears to be stable because it has a poor branch point, which may  
15 allow the 5' end of the intron to bind but which is not released by the de-branching enzyme. The LAT intron thus remains in the lariat structure so that its 5' end is not exposed and thus the intron is stable.

The invention is to use either the LAT's branch point sequences, or any branch point sequences which may result in a binding of the  
20 intron's 5' end but not its release. By constructing a simple splicing cassette, with the consensus splicing signals used for the splice donor site, which specifies the 5' end of the intron; the polypyrimidine tract and the splice acceptor site, which specifies the 3' end of the intron; and by utilizing an appropriate branch point, which, as discussed in the previous  
25 sentence, could be LAT's branch point, or any other sequence which fulfills the functions of forming a lariat and not debranching the lariat, the invention allows the construction of any stable intron by insertion of sequences between the consensus splice donor site and the branch point. The normal splicing of an intron from between two exons is  
30 diagrammed in Figs. 1A and 2A. A simple RNA sequence with two exons surrounding an intron is spliced as shown by the arrows in Fig. 1A, using the consensus splice donor and acceptor signals. Fig. 2A shows



that the two exons are joined together and a lariat is formed by the 5' end of the intron binding to the consensus branch point sequence of UACUAAC. The debranching enzyme recognizes this structure and debranches the lariat, exposing the 5' end of the intron, which is then degraded. Figs. 1B and 2B show the case for the invention. The same simple RNA structure is depicted, with two exons and an intron, but the branch point sequence of the intron is different from the UACUAAC, and is denoted by a series of X's, since LAT's sequence, or other sequences may be inserted here, so long as they form a lariat but do not debranch. As shown in Fig. 2B, the exons are joined and the lariat is formed, but the lariat cannot be debranched and so it is not degraded in a rapid manner, from its 5' end. For example, by using the LAT RNA's branch point nucleotides, artificial introns can be constructed to contain a wide variety of antisense RNAs, to make stable and effective antisense inhibitors of mRNA function.

For example, this can be used, but is not limited to, the insertion of HIV nucleotides in an opposite strand direction for the transcription and stabilization of HIV antisense RNA molecules. For example, one could make lariats containing important and long stretches of RNA antisense to HIV mRNA. Fig. 3A schematically presents the structures of HIV mRNAs. It is preferable that an antisense RNA be chosen such that it will bind most, and more preferably all, of the mRNAs, this would be the case for the antisense constructs A, B, C and D (schematically shown in Fig. 3B, and discussed further in EXAMPLE 1, below). The antisense RNAs are preferably complementary to mRNAs of Tat, nef, and/or rev; since all three proteins start the cascade for viral replication. Tat protein activates the LTR to make transcription of the HIV genes, nef is responsible for HIV pathogenicity, and rev protein is the first HIV protein to be made. Properly inserted into stem cells, these lariats could render the CD4 positive cells either resistant or nonpermissive to HIV infection. Thus, the invention allows the immune system of an HIV-infected, especially an AIDS, patient to be reconstituted. This approach

- is also attractive for newborn infants with AIDS because newborns have in their umbilical cord, stem cells which are more permissive to a retrovirus vector than are adult stem cells. Newborn stem cells have been successfully infected with a retrovirus vector expressing the
- 5 Adenine Deaminase gene, permitting at least a partial cure for two newborns with Severe Combined Immunodeficiency Disease.

- The antisense RNAs of the present invention would accumulate in the nucleus, where their antisense effect would be greater than in the cytoplasm. They could also be very long, and accumulate to high levels.
- 10 For HIV, the advantages accrued would be such that antisense RNAs could inhibit many different strains, since all strains of HIV would share enough basic sequences to bind to the antisense intron. Due to the longer length of the antisense RNAs, there would be no possibility of accumulating resistant viral strains because the antisense RNA will still
- 15 bind to a target RNA which has developed several mutations. The preferred length of the antisense RNA will depend on its specificity, stability, and efficacy in inhibiting its target mRNA. Generally, but not necessarily, the antisense RNA is more than about 100 bases in length.

- The antisense oligonucleotides of the present invention have the
- 20 advantage of being present in the cell nucleus. By being present in the nucleus, the antisense RNA can be designed to block undesirable protein expression at an early stage. For example, to prevent the protein expression of microorganisms which splice their RNA, the antisense RNA can bind to their RNA in the nucleus, interfering with the splicing of the
- 25 RNA and blocking its transportation to the cytoplasm, thus preventing the formation of mRNA and its translation a protein. This approach is more effective than the prior art approach of interfering with the mRNA in the cytoplasm because once the ribosomes in the cytoplasm bind to the mRNA, the antisense RNA will be prevented from binding and inhibiting
- 30 the translation of the mRNA. Further, the nucleus is smaller than the cytoplasm, thus allowing for closer proximity and faster interaction

between the antisense RNA and the RNA. Lesser amount of oligonucleotides are needed for a smaller space.

Further, the antisense RNA of the present invention also have the further advantage of being capable of replicating with the dividing cells  
5 and are thereby passed on to progeny cells.

In detail, applicant has developed a novel antisense approach which is based on the mechanics of RNA splicing. RNA Polymerase II is responsible for transcribing genes in eukaryotic cells, and it makes a long, primary transcript which contains both exons and introns. When  
10 two exons are to be spliced together, the RNA is cut at the splice donor and at the splice acceptor sequence. The 5' end of the intron then loops around and covalently binds to the branch point, which is near the 3' end of the intron. This linkage produces a structure called a lariat, because it is essentially a circle with a small 3' tail. The intron in the lariat form is  
15 stable and is protected from Rnases which normally degrade the RNA from the 5' end. However, once the two exons are joined, a debranching enzyme promotes the release of the lariat, exposing the 5' end of the intron, which is then rapidly degraded. Thus in a normal situation, introns are highly unstable, whereas the mRNA structure of exons has its  
20 5' end protected by a cap structure which is added at the beginning of transcription.

Two naturally occurring examples of stable introns are the LAT of Herpes Simplex Virus, and the T cell receptor B-locus {Farrell, M.J., *et al.*, *PNAS (USA)*, 88:790-794 (1991); Qian, L., *et al.*, *Nucleic Acid Res.*,  
25 20:5346-5350 (1992)}. These RNAs accumulate as stable introns apparently because they are not debranched and remain in the lariat form. The present invention presents a cassette and its construction in which a generic promoter is used to drive an intron which is spliced, forms a lariat, and then is not debranched. By placing sequences of  
30 choice into the intron, in place of the LAT or T cell receptor sequences, the present invention provides stable introns of any DNA sequence. In a preferred embodiment of the invention, these sequences are positioned

such that they are antisense to the sequences of a given gene, thus creating stable introns which are antisense to any gene of interest. The critical requirement here, the invention, is that at the branch point sequences the general cassette has DNA sequences which allow the lariat to form, but which are not a good substrate for the debranching enzyme. Thus either the LAT, or the T cell receptor gene, or any other sequence motif which fulfills these two requirements could be used to create a stable intron.

One practical example of how such an antisense RNA might be used commercially is in the AIDS field. In the preferred embodiment of the invention, a cassette is inserted into a viral vector such as a retrovirus. The cassette preferably contains a promoter which functions in CD4 positive T cells, and which is used to drive stable introns containing antisense molecules to HIV genes. By inserting such viral vectors into stem cells of the immune system, the AIDS patient will be populated through cell division of the stem cells with immune cells carrying HIV antisense RNAs in their nuclei. Such cells may get infected with HIV, but when the virus tries to replicate, the newly made mRNAs of the virus will be inhibited by the antisense RNA introns and will be inactivated. Such an antisense system would create an immune population which is resistant or nonpermissive for HIV replication and would result in either a substantial decrease in HIV virus in the patient or a virtual clearance of the virus from the system.

Generally but not necessarily, the efficacy and safety of the cassettes are first tested and screened in *in vitro* cell system, and the viral vectors carrying the chosen cassettes are tested in the targeted plant or animal. When a human is the target, the viral vector is tested in a scientifically acceptable animal model before testing in human clinical trials. The tests may be carried out using methods known in the art or later developed for the particular target organism, disease and disease agent.

Retroviral vectors are the preferred vectors of this invention, though other viral vectors may be used, such as adenoviral vectors. Though adenoviral vectors have the advantage of not requiring dividing cells for transfection, they have a disadvantage in that they do not  
5 integrate into the genome, possibly making it more difficult to derive stable cell lines. Adeno-associated viral vectors might also be used but have the disadvantage of a smaller packaging limit than retroviral vectors.

The retroviral vector can be any that are known in the art.

10 Retroviruses to be adapted for use in accordance with this invention can be derived from many avian or mammalian hosts. However, a requirement for use is that the virus be capable of infecting cells which are to be the recipients of the new genetic material (the cassettes of the present invention) to be transduced using the retroviral vectors.

15 Examples of retroviruses include avian retroviruses, such as avian erythroblastosis virus, avian leukosis virus, avian myeloblastosis virus, avian sarcoma virus, Fujinami sarcoma virus, spleen necrosis virus (SNV), and Rous sarcoma virus (RSV). Non-avian viruses include: bovine leukemia virus; feline retroviruses such as feline leukemia virus or  
20 feline sarcoma virus; murine retroviruses such as murine leukemia virus, mouse mammary tumor virus, and murine sarcoma virus (MSV); rat sarcoma virus; and primate retroviruses such as human T-cell lymphotropic viruses 1 and 2, and simian sarcoma virus. Many other suitable retroviruses are known to those skilled in the art. A taxonomy of  
25 retroviruses is provided by Teich, in Weiss, *et al.*, eds., *RNA Tumor Viruses*, 2d ed., Vol. 2, Cold Spring Harbor Laboratory, New York, pp. 1-16 (1985). Particularly preferred retroviruses for use in connection with the present invention are the murine retroviruses known as Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMSV)  
30 and Kirsten murine sarcoma virus (KiSV). The MoMSV genome can be obtained in conjunction with a pBR322 plasmid sequence pMV (American Type Culture Collection, Rockville, Maryland, USA, ATCC 37190), while a

cell line producer of KiSV in K-BALB cells has been deposited with the American Type Culture Collection. A deposit of a plasmid (pRSVneo) derived from pBR322 including the RSV genome and a neo marker is available as ATCC 37198. A plasmid (pPB101) comprising the SNV  
5 genome is available as ATCC 45012. For example, a retroviral vector may be constructed so as to lack one or more of the replication genes such as gag (group-specific antigen), pol (polymerase) or env (envelope) protein encoding genes. The resulting recombinant retrovirus would thus be capable of integration into the chromosomal DNA of an infected host  
10 cell, but once integrated, be incapable of replication to provide infective virus, unless the cell in which it is introduced contains another proviral insert encoding functionally active trans-acting viral proteins. Methods for producing infectious but replication deficient viruses are known in the art such as described in Mann, *et al.*, *Cell*, 33:153 (1983) and Miller, *et al.*,  
15 *Mol. Cell Biol.*, 6:2895 (1986), hereby incorporated by reference in their entirety. The CMV immediate-early promoter, or other promoters known in the art, such as a T cell specific promoter, a human retrovirus LTR, or a promoter which functions solely in CD4 positive T cells will be used in these vectors.

20 The present invention thus allows for cell and gene therapies which genetically modify cells which are the target of a pathogen or disease. The cells to be modified are preferably precursor or parental cells. The target tissue or cells may also be surgically removed, the cells expanded, genetically modified to carry the antisense cassette, and  
25 reinjected into the patients.

Bone marrow may be modified by either lipofectin or electroporation to introduce the DNA cassette, followed by drug selection to G418 or hygromycin resistance or selection tailored to other known selection markers, also encoded on the cassette. The genetically  
30 modified bone marrow may then be transplanted into a patient to reconstitute the patient's blood and immune cells. Bone marrow

transplantation is an established procedure following high-dose cancer chemotherapy.

Alternatively, to reduce the need for invasive and costly bone marrow collection procedures, the stem cells may be isolated from peripheral blood and genetically modified *ex vivo*. The genetically modified cells, e.g., bone marrow cells such as hematopoietic stem and progenitor cells, are then isolated and expanded. Methods known in the art may be used, or modified without undue experimentation, to introduce the genetically modified cells into a patient. In the preferred embodiment, these genetically modified hematopoietic stem and progenitor cells will multiply and eventually differentiate into the various cell types to populate and replenish the blood and immune systems. Such a procedure will make autologous and allogeneic transplantation amenable to a larger patient population. A smaller therapeutic dose of concentrated stem cells would reduce the amount of material collected, stored and reinfused into the patient, lessening many of the side effects associated with bone marrow transplantation and immunotherapy.

Specific cell populations may be isolated using methods known in the art, such as a monoclonal antibody-based system. For example, Baxter Healthcare Corp. markets its Isolex® 300 magnetic cell separator system in Europe for stem cell isolation and reinfusion following cancer chemotherapy. Collect™ (Biotex Laboratories, Inc., Edmonton, Canada) negative-selection system yields an enriched T-cell population from peripheral blood or bone marrow samples and can separate CD4+ or CD8+ T-cell subpopulations. These cells are then expanded, genetically modified, and later reinfused into the patients. The cells can also be stored for later use. A genetically modified cell lines may also be established.

The use of this antisense system is not limited to HIV infections. Any cellular or viral RNA may be inhibited by the antisense approach of the present invention. For example, many other animal viruses could be inhibited by viral-specific antisense RNAs. These include, but are not

limited to, Hepatitis B & C, Herpesviruses such as Epstein-Barr virus, Varicella Zoster Virus (the chickenpox virus), Cytomegalovirus, and Herpes Simplex Virus. For example, liver cells might be made resistant to hepatitis viruses by injecting a viral vector into the portal vein. This virus vector should contain a promoter which functions well in liver cells, and should deliver an intron which is antisense to crucial genes in the hepatitis virus. This would make the liver tissue full of hepatocytes which are resistant to hepatitis virus. Epstein-Barr virus and Cytomegalovirus might be inhibited by a stem cell infection as used in the HIV model, whereas Herpes Simplex virus and Varicella Zoster Virus might be inhibited by infection of neurons harboring these viruses with viral vectors which synthesized the appropriate antisense introns which would then inhibit reactivation of these viruses from the latent state.

In addition, many plant viruses may be inhibited by incorporating antisense molecules directly into the plant tissues. These include plant and tomato viruses, turnip yellow mosaic virus, and many other viruses which cause plant disease. In plants, for example, a plant tissue specific or tissue active promoter would be used to introduce an antisense RNA, using the present invention in place of current antisense technology for plants or using known or later developed techniques for the delivery and expression of exogenous DNA in plant cells, such as described in Hamilton, A. J., *et al.* {*Current Topics in Microbiology & Immunology*, 197:77-89 (1995)}. The basic invention would cover all of these, since the splicing apparatus is the same. Other uses in the plant world would include the blocking of certain genes which would lead to better agricultural products. A prime example is the antisense inhibition of fruit ripening genes, such as in tomato {Hamilton, A. J., *et al.*, *Current Topics in Microbiology & Immunology*, 197:77-89 (1995)}.

Other uses of the present invention include antisense inhibition for treating cancers, e.g., such as acute and chronic myelogenous leukemia, gliomal tumors, ovarian cancers and esophageal cancers. For example, the present invention provides for stable introns containing antisense



RNAs against specific RNAs accumulating in cancer cells or to inhibit a protein function that is responsible for the cancer. For example, there are antisense mRNAs made against TGF- $\beta$  ("TGF" denotes tumor growth factor) in brain tumors. These tumors are surgically removed, the

5 antisense RNAs are inserted into the tumors by lipofectin transfection of the plasmid cassette, and the cells are made resistant to G418 or other selection marker (e.g. a drug resistant marker), also carried on the plasmid cassette. Resistant tumor cells expressing the TGF- $\beta$  are injected back into the patient, where the cells do not grow aggressively,

10 because of the lack of TGF- $\beta$  mRNA, but they do help mount an immune response to not only themselves, but more importantly, to the few remaining cells not surgically removed which do express TGF- $\beta$ . Some leukemia cells might be attacked through their production of a unique mRNA, such as in the Philadelphia chromosome rearrangement, where

15 the Bcl-Abl fusion produces a new mRNA which can be inhibited by a viral vector of the present invention expressing the appropriate antisense RNA. The recombinant vectors of the present invention, such as plasmid proviral DNA, can be directly injected into an animal or human using methods known in the art, e.g. by microinjection or particle bombardment

20 such as by gene gun {see e.g., Yang, N. *et al.*, *Gene Therapeutics*, J. A. Wolff, ed., Birkhauser, Massachusetts, USA (1994)}. Preferably, the recombinant vectors are solubilized in physiologically acceptable carriers. Other methods known in the art may also be used. For example, direct *in vivo* gene transfer may be also be achieved with formulations of DNA

25 encapsulated in liposomes, DNA entrapped in proteoliposomes containing viral envelope receptor proteins {e.g., using the method disclosed in Nicolau, C., *et al.*, *PNAS (USA)*, 80:1068 (1983), hereby incorporated by reference in its entirety}, calcium phosphate-coprecipitated DNA {e.g., using the method disclosed in Benvenisty, N.,

30 *et al.*, *PNAS (USA)*, 83:9551 (1986), hereby incorporated by reference in its entirety}, and DNA coupled to a polylysine-glycoprotein carrier complex {e.g., using the method disclosed in Wu, G. Y., *J. Biol. Chem.*,

263:14621 (1988), hereby incorporated by reference in its entirety}. *In vivo* infection with cloned viral DNA sequences after direct intrahepatic injection with or without formation of calcium phosphate coprecipitated DNA may also be used {e.g., using the method disclosed in Seeger, C.,  
5 *et al.*, *PNAS (USA)*, 81:5849 (1984), hereby incorporated by reference in its entirety}. Injection of pure DNA with the promoter-driven cassette directly into an animal or human, such as into their muscle cells, may also be used {e.g., using the method disclosed in Wolff, J. A., *et al.*,  
10 *Science*, 247:1465-1468 (1990), hereby incorporated by reference in its entirety}. Thus, the pure DNA may be delivered intramuscularly, intradermally, intravenously, intraperitoneally, subcutaneously, nasal, and orally. For intraperitoneal and intravenous delivery, lipid is preferably used, as described above, to enhance delivery. Methods such as liposomes may also be used to achieve inoculation of the plasmid  
15 through mucosal membranes {Powell, M. F., *et al.*, *Vaccine Design-The Subunit and Adjuvant Approach*, Plenum Press, New York (1995)}.

In fact, there are over 100,000 genes in the human genome, and many times more than in the plant, animal and viral kingdoms, most of which would be susceptible to antisense inhibition as described here. In  
20 each case, a specific construct with a specific promoter would have to be made.

The examples in this application are presented to illustrate some aspects of the invention, and are not to be construed as limiting the scope of the invention.

25

### EXAMPLES

As a model system for trying out these ideas, applicant has constructed a cassette with the following properties. The promoter is the cytomegalovirus (CMV) immediate-early gene enhancer, which is a very  
30 strong promoter and is commonly used in many systems. The intron contains all the signals of the LAT intron, including the splice donor and acceptor sequences, the branch point and the polypyrimidine tract.

Inserted upstream of the branch point is a single Bgl II restriction site, and into that site applicant has cloned four different HIV DNA fragments ranging in size from 500 bases to 1400. These HIV sequences were placed in the antisense direction with respect to the orientation found on the HIV genome, so that introns were spliced which were antisense to HIV mRNAs. In transient transfection assays, applicant has introduced these four plasmids into rabbit skin cells by lipofectin transfection, and the cells were allowed to synthesize RNA for two days. RNA extracted from these cells and analyzed by Rnase protection assays showed that in all four cases the introns were stable.

### EXAMPLE 1

#### CONSTRUCTION OF THE CASSETTES

##### METHODS

As a model system for trying out these ideas, applicant has constructed a cassette with the following properties. The promoter is the CMV immediate-early gene enhancer, which is a very strong promoter and is commonly used in many systems. This plasmid, pCMVMIEP, was kindly provided by Dr. Peter Ghazal of Scripps Research Institute, La Jolla, California, USA {Ghazal, P., *et al.*, *J. Virol.*, 65:2299-2307 (1991)}, and has the following structure.

CMV enhancer .... Cap site..Bam HI..LacZ gene

Two oligonucleotides were purchased from Operon (Alameda, California, USA) with the following structure:

615A: 5' GATCCAGGTAAGCCTAGATCTAGGGACATGTTTCATGCCTTCTTTTCTAG,

615b: 5' GATCCTAGAAAAAGAAGGCATGAACATGTCCCTAGATCTAGGCTTACCTG.

These oligonucleotides were mixed, heated to 95°C, and annealed overnight at room temperature. The mixture was then used to clone into the Bam HI site of pBluescript SK<sup>+</sup> (Stratagene, La Jolla, California, USA), put into competent *Escherichia coli* DH5 $\alpha$  cells, and plated on

ampicillin plates with X-gal as a substrate. White colonies were screened by minilysate for the presence of the Bgl II site, and recombinant clones were sequenced to verify the correct nucleotide structure. This plasmid, pLTF615, contains on a Bam HI fragment, the splice donor sequence:

- 5 AG:GT; a Bgl II site AGATCT; a mutated branch point from the LAT region AGGGACA; a polypyrimidine tract and a splice acceptor site, TTCTTTTCTAG:G. The splices between the exon and intron are indicated by colons. What is critical in this construction is the nonconsensus branch point, which allows the lariat to form but does not  
10 allow the debranching enzyme to linearize this RNA after splicing is completed. Other branch points could be used in place of the one utilized here, so long as they form a lariat which is not debranched.

- The Bam HI fragment from pLTF615 was cloned into the Bam HI site of pCMVMIEP and the orientation was verified by restriction analysis.  
15 This provides the basic backbone cassette, with the Bgl II site in the middle of a stable intron, into which applicant cloned four HIV DNA fragments. The intron contains all the signals of the LAT intron, including the splice donor and acceptor sequences, the branch point and the polypyrimidine tract. These HIV sequences were placed in the antisense  
20 direction with respect to the orientation found on the HIV genome, so that introns were spliced which were antisense to HIV mRNAs.

- Plasmid A is a 1.4kb intron which is antisense to the major splice donor site at 743 bases and is antisense to gag/pol mRNA for another 1200 bases. Plasmid B is a 1.3kb intron which is at the 5' end and is  
25 antisense to TAR, the major splice donor, and part of gag/pol. Plasmid C is at the 3' end of the genome and is a 1.4kb intron antisense to the 3' ends of env and tat/rev/nef mRNAs. Plasmid D is a 500 bp intron which is a smaller subset of sequences found in plasmid C. The antisense constructs for A, B, C, and D are schematically shown in Fig. 3B,  
30 respectively.

A). Plasmid A (pLTF637) is a 1.4kb anti-gag/pol/env intron which also covers the major splice donor region.

- 5 B). Plasmid B (pLTF649) is a 1.3kb anti-gag/pol intron which covers the major splice donor and bridges both sides of the TAR sequence.
- C). Plasmid C (pLTF638) is a 1.4kb RNA antisense to the mRNA sequences of tat/rev and nef as well as part of the larger RNAs for gag, env and vif.
- D). Plasmid D (pLTF646) is a 0.5kb RNA, a subset of RNA C, antisense to tat/rev and nef.

10 These four RNAs were selected to inhibit some of the key early steps in HIV replication, subsequent to its integration into the host chromosome.

#### Transient Assays:

15 Applicant placed four different introns into a cassette driven by the CMV immediate-early enhancer. These plasmids were used to transfect rabbit skin cells, a common cell eukaryotic cell line {American Type Culture Collection, Rockville, Maryland, USA}. Five micrograms of DNA was mixed with 60 microliters of lipofectin (Gibco BRL, Gaithersburg, Maryland, USA) according to manufacturer's instructions and deposited

20 on a 100mm dish of semi-confluent rabbit skin cells. After two days, the RNA from these cells was extracted and purified using a Qiagen Rneasy Total RNA kit (Qiagen, Inc., Chatsworth, California, USA) for use in an RNase protection assay.

#### 25 RNase Protection Assay:

Riboprobes and all RNA procedures were used in the Ambion ribonuclease protection assay kit (Ambion, Inc., Austin, Texas, USA), as per manufacture's instructions. Purified RNA was annealed at 42°C with non-radioactive riboprobes overnight, and digested with ribonucleases in

30 the appropriate buffers. RNA samples, as well as RNA probes, were electrophoresed on a 6% acrylamide-urea gel, according to Ambion, Inc.'s instruction, and transferred as in a Western blot to nylon filter

paper, where the chemical reactions were performed. The final, washed filter was exposed to x-ray film to visualize the bands as shown in Fig. 4.

As shown in Fig. 4, in lane 4, there is the RNA probe for plasmids C and D run by itself. Lane 5 shows the digested probe with  
5 untransfected RNA from rabbit skin cells, and there is no band. Lane 6 shows the RNA from transfection of plasmid #638 (plasmid C) and there is a protected band just below the size of the full length probe. In lane 7, there is RNA from transfection of plasmid #646 (plasmid D) which shows a slightly larger protected band of the correct size. Though antisense  
10 construct D is a subset of C, the protected band should be larger because of the way the probe was made. In lane 8, there is the RNA probe for antisense construct B, lane shows the control RNA and there is some self-annealing of the full length probe. Lane 10 shows the protected band from cells transfected with plasmid 639 (plasmid B),  
15 which is not present in the-control lane.

Thus, plasmids B, C, and D all clearly show protected bands. In the plasmid A transfection, the RNA had to run out longer to see a protected band.

20

## **EXAMPLE 2**

### **DEVELOPMENT AND USE OF THE CASSETTES**

SUPT1 cells, a leukemia cell line infectable by many laboratory isolates of HIV, will be used to isolate cell lines expressing the introns. Applicant is currently testing the drug sensitivity to neomycin and  
25 hygromycin prior to transfecting these cells and selecting resistant cell lines. The stable introns will be tested for their mild toxicity, if any, to these cells. Cell lines containing cassettes will be generated. Applicant has in this laboratory made several cell lines expressing the 2kb LAT intron, and applicant knows of another naturally occurring stable intron,  
30 found in the T cell receptor locus, which is also found to accumulate in dividing cells {Qian L., *et al.*, *Nucleic Acid Res.*, 20:5346-5350 (1992)}. Thus there are two precedents for a stable intron accumulating in the

nuclei of dividing cells. However, it remains theoretically possible that the antisense RNAs applicant has constructed might be mildly toxic to the cells. Plasmid C, for example, might interfere not only with the splice acceptor of HIV, but with that of other cellular mRNAs. It is possible that the CMV enhancer may be too strong, and applicant may have to use a weaker promoter with some constructs. Applicant would make modifications in the constructs as needed, so that applicant will have several constructs which are functional in preventing HIV gene expression *in vitro*. These modifications may include:

- a) Using a weaker promoter than the CMV enhancer;
- b) Putting the intron between the promoter and the neomycin gene, so that neomycin resistance is obtained only if the intron is correctly spliced. This may be useful if the intron is mildly toxic;
- c) Making smaller introns. This might be necessary if the larger ones applicant has constructed are either toxic or not as stable in cell lines as applicant would like; and
- d) Putting two introns into the same transcription unit. One nice feature of this system is that the antisense RNAs are introns. Thus, there is no reason why applicant could not put two or three introns into the same transcription unit. Thus, applicant could use an intron against the 5' end of the HIV genome with another one antisense to the 3' end. This could make the antisense system even more effective.

Following the successful establishment of cell lines, the RNA in such drug resistant cells will be examined by Rnase protection assays (using methods known in the art or as described above) and by Northern blot analysis (using methods known in the art) to confirm the presence in the nucleus of a stable intron. Control cell lines, with introns that are properly debranched, will be used to show that the stability is due to applicant's particular intron structure.

C II lines which express HIV antisense RNA molecules will be infected with laboratory strains of HIV to monitor the antisense inhibition. Applicant will determine the ability of HIV strains to express p24gag (using methods known in the art, such as described in Zack, J. A., *et al.* {*Science*, **240**:1026-1029 (1988)}) as a function of time, using as controls  
5 cell lines which do not express the stable introns.

The long term goal of this work is to identify one or more constructs which are stable in dividing cells and which inhibit HIV gene expression *in vitro*. Following a successful *in vitro* inhibition, applicant  
10 will test the construct or constructs *in vivo*. Applicant will utilize the best antisense constructs from the infected cell line data to construct a retrovirus containing this cassette for insertion into CD34 cells. The transduced CD34 cells are then implanted into SCID mice, using the procedure described in Akkina, R. K., *et al.* {*Blood*, **84**:1393-1398  
15 (1994)}. These mice and control mice will be infected with HIV and the effectiveness of the antisense RNA in this animal model will be determined, using the methods described in Akkina, R. K., *et al.* {*Blood*, **84**:1393-1398 (1994)} and Aldrovandi, G. M., *et al.* {*Nature*, **363**:732-736 (1993)}. These vectors may be further tested and selected in another  
20 animal model for their safety, efficacy and optimal application, using methods known in the art. Finally, the effective vectors are tested and selected in human clinical trials based on their safety, efficacy and optimal application. For example, the vectors are used to transduce the stem cells of an HIV-infected or AIDS patient. The transduced stem cells  
25 are then reintroduced into the patient. The patient is monitored for immunological parameters, clinical signs, viral load and/or other known indicators of HIV presence and/or activities, using methods known to one skilled in the art. The preferred vectors are those which are able to reconstitute the patient's immune system with non-HIV permissive cells,  
30 thereby resulting in an increase in CD4 cells and decrease in HIV viral load in the patient.



All publications and patent applications mentioned in this Specification are herein incorporated by reference to the same extent as if each of them had been individually indicated to be incorporated by reference.

- 5           Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that various modifications and changes which are within the skill of those skilled in the art are considered to fall within the scope of the appended claims. Future technological
- 10   advancements which allow for obvious changes in the basic invention herein are also within the claims.

I claim:

1. A splicing cassette comprising:
  - a) a promoter, capable of expressing RNA sequences in eukaryotic cells;
  - b) a splice donor site downstream from the promoter;
  - 5 c) a splice acceptor site downstream from the splice donor site;
  - d) a branch point sequence between the splice donor and acceptor sites, wherein the branch point can form a lariat but cannot debranch; and
  - 10 e) an antisense sequence inserted between the splice donor site and the branch point sequence.
2. A splicing cassette according to claim 1 wherein the promoter is selected from the group consisting of the CMV immediate early promoter, a T cell specific promoter, a retrovirus LTR, and a promoter that functions solely in CD4 positive T cells.
3. A splicing cassette according to claim 1 wherein the branch point sequence is derived from the herpesvirus LAT sequence or T cell receptor sequences.
4. A splicing cassette according to claim 1 wherein the antisense sequence is complementary to mRNA sequences selected from the group consisting of HIV, Hepatitis B & C, Epstein Barr virus, Varicella Zoster Virus, Cytomegalovirus, Herpes Simplex Virus, and TGF- $\beta$  mRNA sequences.
5. An antisense vector comprising:
  - 1) a transfer vector capable of transducing or transforming eukaryotic cells; and

- 2) a splicing cassett inserted within the transfer vector, the  
5 splicing cassette comprising:
- a) a promoter, capable of expressing RNA sequences in eukaryotic cells;
  - b) a splice donor site downstream from the promoter;
  - c) a splice acceptor site downstream from the splice  
10 donor site;
  - d) a branch point sequence between the splice donor and acceptor sites, wherein the branch point can form a lariat but cannot debranch; and
  - e) an antisense sequence inserted between the splice  
15 donor site and the branch point sequence.

6. An antisense vector according to claim 5 wherein the transfer vector is selected from the group consisting of retroviral, adenoviral, and adeno-associated viral vectors.

7. A cell line comprising, a eukaryotic cell stably transformed by an antisense vector, wherein the antisense vector can express a stable intron, the stable intron comprising, a mutated branch point capable of forming a lariat but incapable of debranching, and an antisense RNA inserted within the lariat.

8. A cell line according to claim 7 wherein the eukaryotic cells is selected from the group consisting of SUPT1, CD34 cells, CD4 cells, hematopoietic stem cells, and hematopoietic progenitor cells.

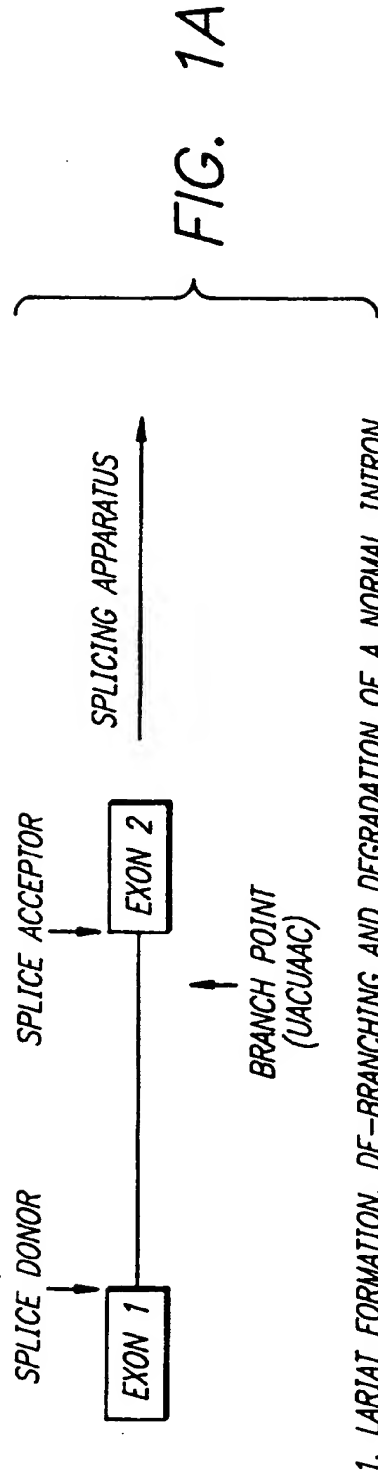
9. A method of gene therapy comprising transforming or transducing eukaryotic cells with an antisense vector, the antisense vector comprising:

- 1) a transfer vector capable of transforming or transducing  
5 eukaryotic cells; and

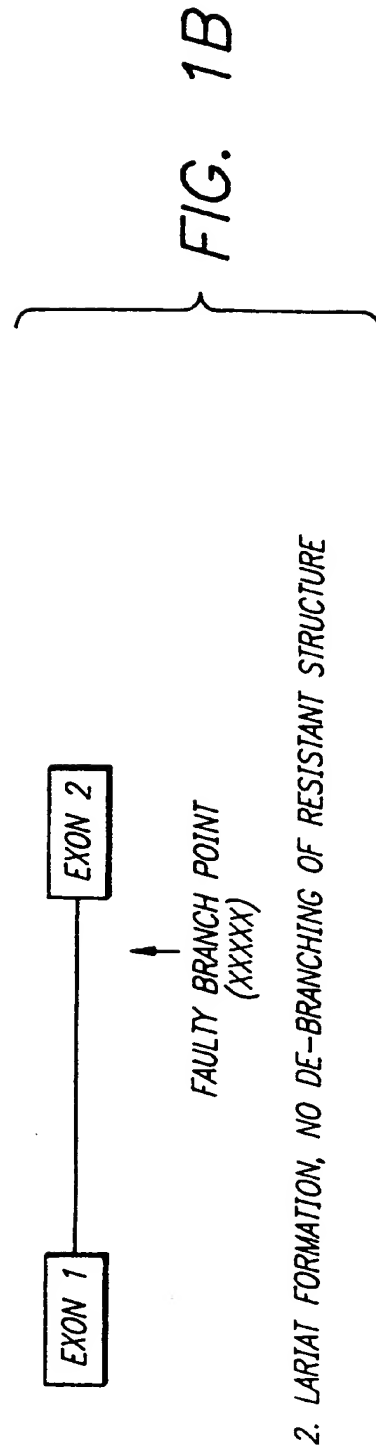
2) a splicing cassette inserted within the transfer vector, the splicing cassette comprising:

- a) a promoter, capable of expressing RNA sequences in eukaryotic cells;
- 10           b) a splice donor site downstream from the promoter;
- c) a splice acceptor site downstream from the splice donor site;
- d) a branch point sequence between the splice donor and acceptor sites, wherein the branch point can
- 15           form a lariat but cannot debranch; and
- e) an antisense sequence inserted between the splice donor site and the branch point sequence.

10. A method of gene therapy according to claim 9 wherein the eukaryotic cells are selected from the group consisting of CD34 cells, CD4 cells, hematopoietic stem cells, hematopoietic progenitor cells, bone marrow cells, liver cells, and neurons.



1. LARIAT FORMATION, DE-BRANCHING AND DEGRADATION OF A NORMAL INTRON



2. LARIAT FORMATION, NO DE-BRANCHING OF RESISTANT STRUCTURE

FIG. 2A

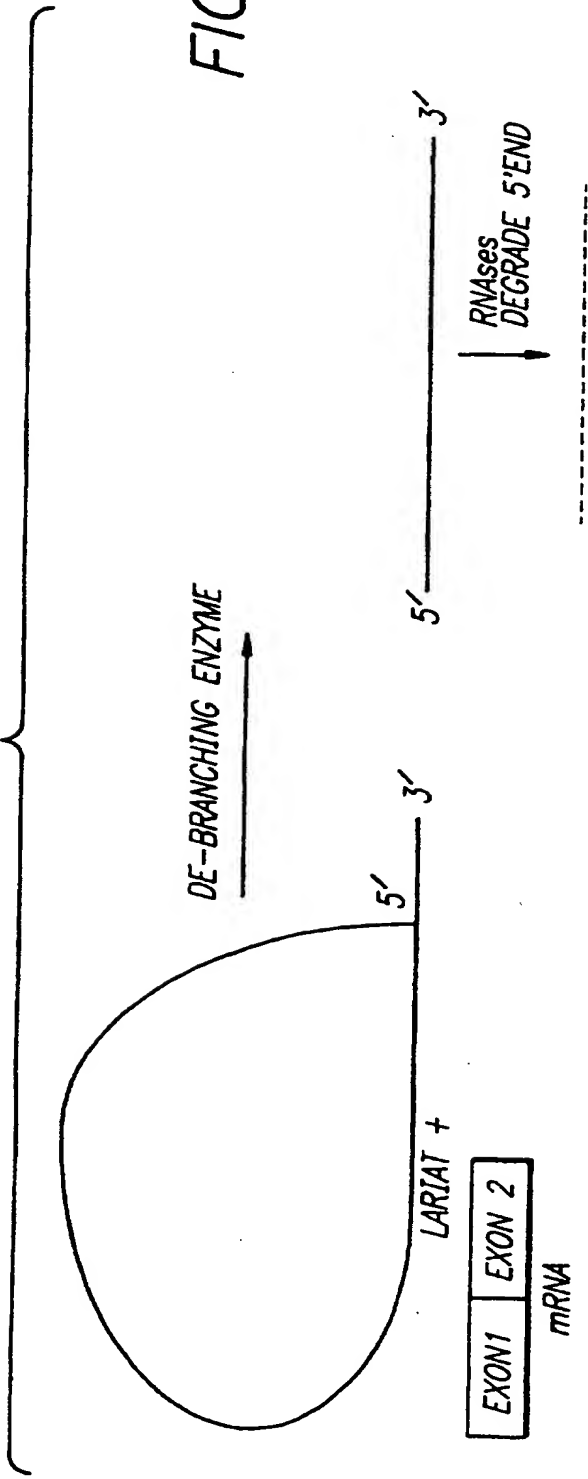
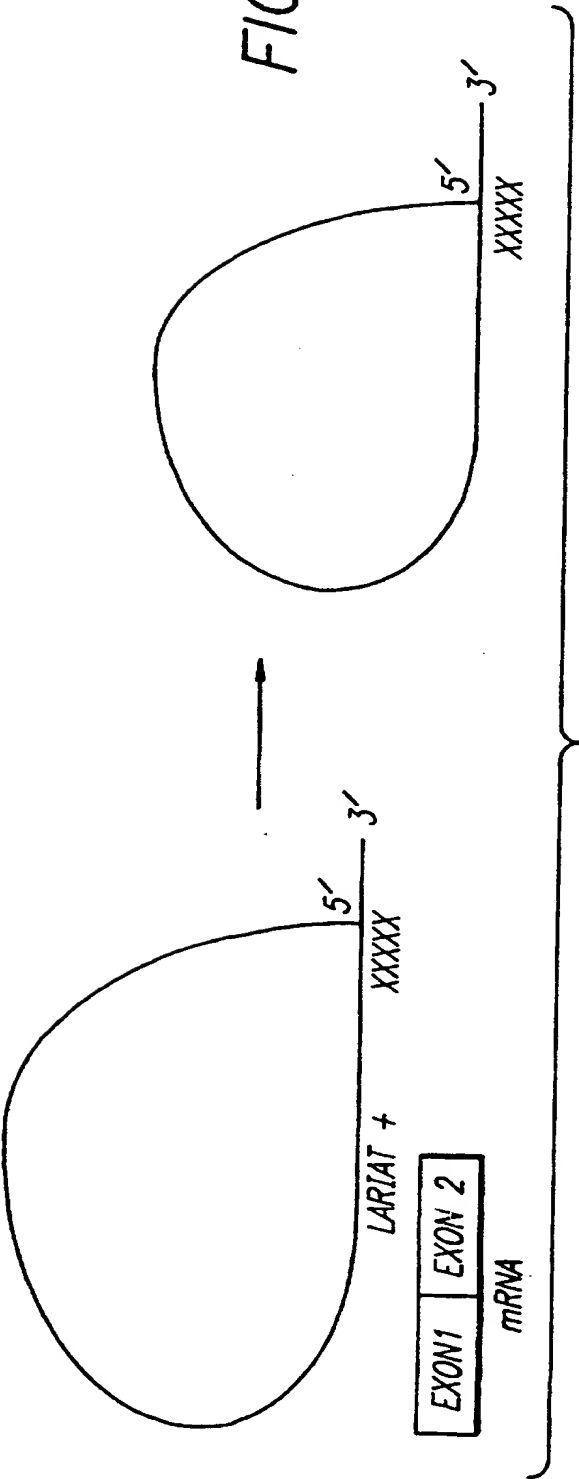
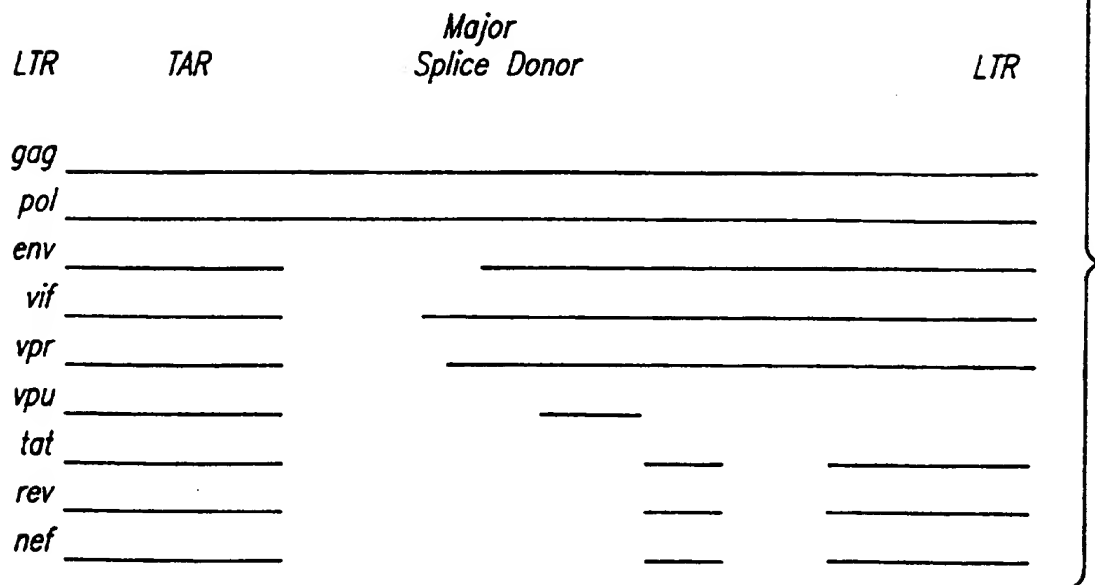


FIG. 2B



3/4

## FIG. 3A

STRUCTURES OF HIV mRNAs*Antisense constructs:*

- A. \_\_\_\_\_
- B. \_\_\_\_\_
- C. \_\_\_\_\_
- D. \_\_\_\_\_
- \_\_\_\_\_

## FIG. 3B

FIG. 4





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/04519**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 48/00; C07H 21/04; C12N 15/63, 15/85

US CL : 435/320.1, 325; 514/44; 536/24.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 325; 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, BIOSIS, EMBASE, WPIDS

search terms: branch point, splice, splice acceptor, splice donor, lariat, mutant, mutation, debranching

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HARRIS et al. Distribution and consensus of branch point signals in eukaryotic genes: a computerized statistical analysis. Nucleic Acids Research. 1990, Vol. 18, No. 10, pages 3015-3019, see entire document.	1-10
Y	JACQUIER et al. RNA splicing and intron turnover are greatly diminished by a mutant yeast branch point. Proceedings of the National Academy of Sciences USA. August 1986, Vol. 83, pages 5835-5839, see entire document.	1-10
Y	SCANLON et al. Oligonucleotide-mediated modulation of mammalian gene expression. The FASEB Journal. October 1995, Vol. 9, pages 1288-1296, see entire document.	1-10

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 MAY 1997

Date of mailing of the international search report

02 JUN 1997

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